

BASAL RELEASE OF ENDOTHELIUM-DERIVED NITRIC OXIDE IN  
THYROPATHOLOGIC RAT AORTA

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A Thesis  
Presented to  
The College of Arts and Sciences  
Drake University

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In Partial Fulfillment  
of the Requirements for the Degree  
Master of Arts

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by  
Lisa C. Frazzell  
January 1994

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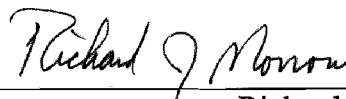
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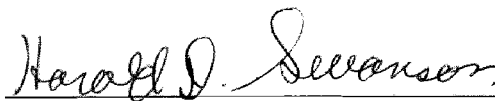
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# BASAL RELEASE OF ENDOTHELIUM-DERIVED NITRIC OXIDE IN THYROPATHOLOGIC RAT AORTA

An abstract of a Thesis by

Lisa C. Frazzell

January 1994

Drake University

Systolic blood pressures, basal metabolic rates, and circulating catecholamine levels were determined for hyperthyroid (TRX), hypothyroid (PTU), and euthyroid control (CON) rats. All three measurements were elevated in TRX animals, while PTU rats were determined to have decreased measurements when compared with CON and TRX rats. Animals were sacrificed by either cervical dislocation or pentobarbital injection followed by thoracic opening to test for sacrifice differences. Three aortic rings from each rat were mounted in environmentally-controlled tissue baths and contracted with different concentrations (10 nM, 100 nM, and 1 mM) of phenylephrine (PE), an alpha-1 adrenoreceptor agonist. At steady state, methylene blue (MB) (10 mM), an endothelium-derived nitric oxide (EDNO) inhibitor, was added and rings were allowed to contract further. At steady state, a high PE dose (10 mM) was added to produce maximum contraction. Irrespective of the sacrifice method, the basal release of EDNO as a percent of the maximum force generated, was PTU>CON>TRX, while the mg of force unmasked by MB was not different. The trend in mg of force produced by PE was TRX>CON>PTU regardless of the initial PE concentration. As the concentration of the initial PE dose was increased, the percent of the total PE plus MB response that was due to MB alone decreased in CON rats. Sacrifice with pentobarbital followed by thoracic opening eliminated the difference between TRX and CON expressed in animals sacrificed by cervical dislocation, while the relationship between CON and PTU was unchanged. Just as thyropathology differentially altered systolic blood pressure, basal metabolic rate, and circulating catecholamine levels, the basal release of EDNO in rat aorta was found to be dependent upon the thyroid state of the animal and the initial agonist-induced tone.

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## INTRODUCTION AND REVIEW OF LITERATURE

Thyropathology has been reported to produce changes in agonist-induced vascular contractility in the rat aorta, but not with absolute consistency. The hyperthyroid state has been reported by some researchers to reduce vascular smooth muscle (VSM) contractility in response to adrenergic agonists (Coville and Telford 1970; Fox et al. 1985), whereas Hawthorn et al. (1988) found contractility to be unchanged. No change, slight decreases, or slight increases in vascular contractility have been reported in hypothyroidism (Coville and Telford 1970; Fox et al. 1985; Rahmani et al. 1987; Hawthorn et al. 1988; Gunasekera and Kuriyama 1990). Changes in blood vessel adrenoreceptor numbers are also reported to be associated with thyropathology. Gunasekera and Kuriyama (1990) found that alpha adrenoreceptor density decreased and beta adrenoreceptor density increased in hyperthyroid rat aorta while hypothyroidism produced opposite effects in adrenoreceptor densities. This is consistent with an earlier review dealing with nonvascular tissue in which hyperthyroidism is associated with increased BAR activity and, less consistently, decreased AAR activity, while the opposite effects are seen in the hypothyroid state (Bilezikian and Loeb 1983).

While these receptor changes are well accepted, the extent to which thyropathology induces changes in plasma catecholamine levels is unclear (Christensen 1973; Coulombe et al. 1976b; Coulombe et al. 1977; Tsujimoto et al. 1987). Blood-borne catecholamines are a major determinant of vascular compliance and the regulation of blood flow (Vanhoutte 1989), yet, studies of thyropathologic humans have reported no change in circulating catecholamine levels, and have explained observed cardiovascular changes through altered receptor densities (Christensen 1973; Coulombe et al. 1976a, 1976b, 1977). Circulating catecholamine levels have not been reported for thyropathologic rats. Accordingly, one

goal of this study was to determine if changes in contractility occurred in thyropathologic rat aorta and, if so, were those accompanied by changes in circulating catecholamine levels.

More than a decade ago, Furchgott and Zawadzki (1980) discovered that acetylcholine (ACh) elicits an endothelium-dependent arterial relaxation, which was attributed to the release of an endothelium-derived relaxing factor (EDRF). EDRF is now known to be endothelium-derived nitric oxide (EDNO), a short-lived soluble agent released by the aortic endothelium in response to a variety of agonists, including serotonin (5-HT) (Furchgott 1983). EDNO acts by inducing an increase in cellular levels of cyclic guanosine monophosphate (cGMP) and causing relaxation of the VSM (Rappaport and Murad 1985). The EDNO inhibitor, methylene blue (MB), prevents nitric oxide (NO) action on VSM cells by binding to the heme subunit of soluble guanylate cyclase, thereby preventing the increase in cGMP and relaxation of the cell (Rappaport et al. 1985).

Aside from agonist-induced EDNO release, the basal influence of EDRFs in rat aorta has been established (Griffith et al. 1984; Lacolley et al. 1991; Long and Stone 1985; Rubyani et al. 1985). Dainty et al. (1990) used PE to induce initial tone in isolated rat aorta, then added ACh and monitored relaxation of the vessel. They found that as the initial PE dose, and therefore the tone of the vessel, increased, the sensitivity to ACh decreased (in gram weight), indicating a reduced agonist-induced NO release. Dainty et al. (1990) also found that responses to PE were greater in rings denuded of endothelium than in rings with an intact endothelium, presumably due to the elimination of EDNO effects in the denuded rings. However, it is unknown whether the effect of basal NO, rather than ACh-induced EDRFs, is stable under all conditions of contractile tension, or is dependent upon the degree of agonist-induced tone in the aorta, as ACh-induced EDRF release is. Therefore, a second goal of this study was to assess the basal release of EDNO under different degrees of phenylephrine (PE)-induced tone by the addition of MB, which

prevents the effect of NO on the VSM cells without disrupting or activating the endothelium.

Many laboratories have evaluated changes that accompany thyropathology in the rat, including changes in blood pressure and basal metabolic rate (BMR). Blood pressure (BP) has been reported to be elevated in hyperthyroidism and lowered in hypothyroidism, when compared to euthyroid animals (Field et al. 1973; Rioux and Berkowitz 1977). Likewise, BMR is increased in the hyperthyroid state and decreased in the hypothyroid state, when compared with euthyroid rates (Guyton 1991). While the effects of thyropathology on blood pressure and BMR are known, the effects of thyropathology on basal EDNO release, a parameter that could influence blood pressure, have not been reported. Thus, a third goal of this study was to determine the effects of thyropathology on basal EDNO release.

Laboratories involved in the study of thyropathology and vascular endothelium use various sacrifice methods prior to harvesting blood vessel segments. In many laboratories rats are anesthetized prior to vessel harvesting (Lin and Nasjletti 1991; Lüscher and Vanhoutte 1986; Lüscher et al. 1987; Mortensen et al. 1990; Rinaldi and Bohr 1989; Wu and Bohr 1990). Other laboratories use cervical dislocation or similar methods with no anesthesia, which might trigger catecholamine release (Stratton and Morrow 1991; Dainty et al. 1990; Sufka et al. 1990). Many discrepancies are found between data collected from different laboratories, a fact that may be attributed to the use of different sacrifice methods. Accordingly, a fourth goal of this study was to determine whether sacrifice methods might contribute to some of the discrepancies observed between data reported by different experimenters.



## **MATERIALS AND METHODS**

### **Experimental animals**

The subjects were 75 g male Sprague-Dawley rats. Animals were maintained two per cage on a 12 hour light/dark cycle with Purina rat chow and water available ad libitum. Three groups were prepared over a period of two weeks. One group was rendered hyperthyroid (TRX) by daily intraperitoneal injection of 200 mg of L-thyroxine in a 0.2 ml volume of isotonic vehicle (3 ml 95% ETOH, 3 ml 0.01% NaOH, 24 ml 0.9% NaCl). A second group was rendered hypothyroid by the administration of 0.1% 2-thiouracil (PTU) in the drinking water. A third euthyroid control (CON) group was also maintained. To control for handling and injection effects, both the PTU and CON rats were handled and sham injected with vehicle alone daily.

### **Verification of pathologic induction**

The basal metabolic rate (BMR) of each rat, later sacrificed by pentobarbital injection followed by thoracic opening, was measured by indirect calorimetry following the two week induction of thyropathology. Students of Biology 129 (Mammalian Physiology) completed the indirect calorimetry measurements. Elevated BMR was taken as evidence of a hyperthyroid state while depressed BMR was indicative of a hypothyroid condition. The BMRs of rats later sacrificed by cervical dislocation were not measured.

### **Systolic blood pressure analysis**

The systolic blood pressure of each rat, later sacrificed by cervical dislocation, was measured by Karen Kurek (undergraduate assistant) using an indirect tail cuff sphygmomanometric method (NARCO Bio-Systems, Houston, Texas). Before blood pressures were recorded the animals were allowed to adapt to the housing unit and blood pressure cuff through several inflation-deflation cycles. Three additional cycles were then completed and the average of these three recordings was determined to be the systolic

pressure. The blood pressures of rats later sacrificed by anesthesia and thoracic opening were not measured.

### **Catecholamine analysis**

Blood samples (1.5 ml) removed from the thoracic cavity of rats sacrificed by anesthesia and thoracic opening, were transferred to heparinized tubes and centrifuged for five minutes. The supernate was removed from each sample and stored at -70°C until the time of analysis. Analysis was performed by Dr. Dean Hoganson using high pressure liquid chromatography (HPLC) (Beckman, Berkeley, CA). Each sample was assayed for levels of the circulating catecholamines norepinephrine and epinephrine.

### **Dissection and mounting of aortic ring segments**

Rats were sacrificed on day of use by either intraperitoneal sodium pentobarbital injection (40 mg/kg body weight) followed by thoracic opening or cervical dislocation with no anesthesia. A total of 30 rats (10 per treatment group) were sacrificed by pentobarbital injection followed by thoracic opening, while 36 rats (12 per treatment group) were sacrificed by cervical dislocation. The thoracic cavity was opened and an incision was made in the heart of rats sacrificed with anesthesia to assist in the removal of 1.5 ml of blood for the analysis of catecholamines. The thoracic aorta was excised from rats in both sacrifice groups, placed in oxygenated buffered physiologic saline solution (PSS), and dissected free of connective tissue. Three ring segments, each approximately 2 mm in length, were cut from each aorta and mounted on L-shaped chromium wire hooks. The lower hook was fixed while the upper hook was attached to a Grass FT-03D force transducer mounted on a micromanipulator. Each ring was suspended vertically in an individual tissue chamber containing 6 ml of pH adjusted (7.4), temperature controlled (37 °C) PSS bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> (Figure 1). The PSS was a modified Krebs-bicarbonate solution with the following millimolar composition: NaCl,

115.0; KCl, 5.0; MgCl<sub>2</sub>, 1.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25.0; CaCl<sub>2</sub>, 2.5; EDTA, 0.026; and glucose, 11.0.

### **Measurement of contractility**

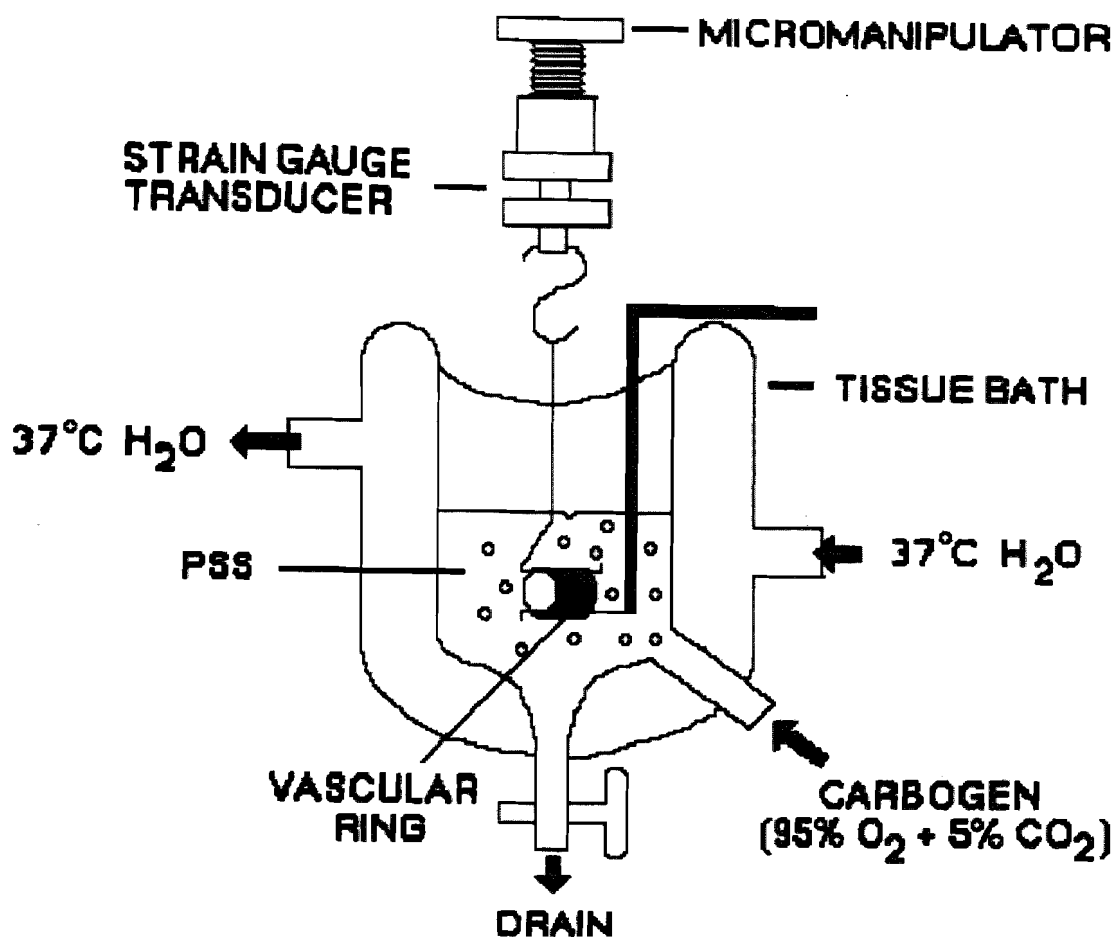
After the three SensorMedic R511 Dynographs were calibrated (2 cm pen displacement set equal to 2 g of force), the ring segments were allowed to equilibrate for two hours at a preload tension of 1 g, found to be an optimal tension in this laboratory. During this time the PSS was changed at 15-minute intervals and tension was maintained at 1 g. Following the equilibration period the rings were contracted by replacement of the PSS with 6 ml of isosmotic high potassium PSS (55 mM) to check viability of the rings. After a contraction was achieved the high potassium PSS was replaced twice with 6 ml applications of normal PSS and the rings were allowed to relax to baseline. Each of the three rings was then contracted with a different concentration of phenylephrine (PE); the first with 10 nM (low), the second with 100 nM (medium), and the third with 1 mM (high) PE. These three PE concentrations were chosen because they are located on the linear portion of concentration-response curves determined in this laboratory. When a maximal contraction was reached, 10 mM methylene blue (MB), an EDNO inhibitor, was added to unmask any contractile tension normally suppressed by basal EDNO. Finally, each ring was exposed to a high concentration of PE (10 mM) to determine the maximal contraction possible for that agonist (Figure 2).

### **Drugs**

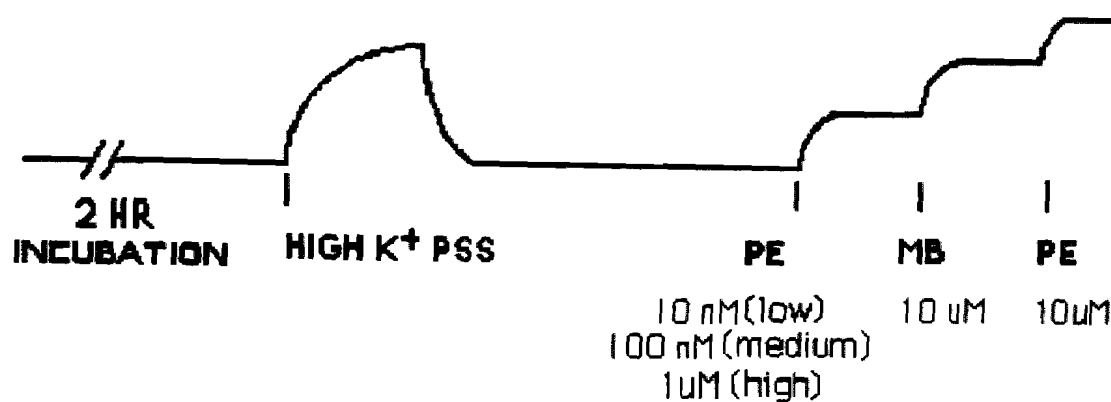
Drugs used were L-thyroxine (Sigma T-2376), 2-thiouracil (Sigma T-7750), sodium pentobarbital (Sigma P-3761), phenylephrine (Sigma P-6126), and methylene blue (Fisher M-291).

### **Statistical analysis**

The experimental values were expressed as the mean  $\pm$  the standard error of the mean (SEM). Statistical significance was determined using the two-tailed Student's t-test for paired and unpaired samples or analysis of variance. When the F value for measurement x treatment interaction from the analysis of variance was significant, individual pairwise comparisons were made using the least significant difference test. In all cases,  $p < 0.05$  was considered significant.



**Figure 1.** Each aortic ring segment is attached to a transducer and micromanipulator at the top and a fixed wire at the bottom. The ring is then suspended in an environmentally-controlled tissue bath. Drug additions are injected directly into the PSS.



**Figure 2.** The protocol as visualized on the dynograph recorder is shown above. Following a two hour incubation period that allowed for vessel equilibration, a high potassium PSS was added to insure ring viability. Each ring was then contracted with a PE dose, followed by MB, used to unmask the force normally prevented by basal EDNO release. Finally, a high PE dose was added to each ring to reach a maximal PE response.

## RESULTS

### Basal metabolic rate

Basal metabolic rates ( $\text{cal/hr/m}^2$ ) were determined for five animals from each of the three treatment groups. These animals were later sacrificed by anesthesia and thoracic opening. Rates were measured by indirect calorimetry and expressed as the mean  $\pm$  SEM (Table 1). PTU showed a decreased BMR ( $46 \pm 4$ ) when compared to CON ( $58 \pm 4$ ), while TRX demonstrated an increased BMR ( $76 \pm 4$ ). The metabolic rates for CON, PTU, and TRX represented statistically different groups.

### Systolic blood pressure

Systolic blood pressures (BP) (mmHg) were measured indirectly by tail cuff sphygmomanometry on rats later sacrificed by cervical dislocation. Values were expressed as the mean  $\pm$  SEM (Table 1). PTU ( $n=8$ ) showed a decreased systolic blood pressure ( $97.7 \pm 5.4$ ) when compared with CON ( $n=8$ ) ( $123.2 \pm 7.1$ ), while TRX ( $n=10$ ) showed an increased systolic blood pressure ( $163.2 \pm 8.5$ ). Based on systolic pressure, animals within the three thyroid groups were significantly different from one another.

### Catecholamine analysis by high pressure liquid chromatography

Plasma norepinephrine (NE) and epinephrine (EP) levels (pg/ml) were determined for rats sacrificed by anesthesia and thoracic opening. Blood was obtained at the time of sacrifice from animals in each treatment group and catecholamine levels were determined by HPLC and expressed as the mean  $\pm$  SEM (Table 1). PTU ( $n=9$ ) showed a decreased level of circulating NE ( $394 \pm 71$ ) when compared with CON ( $n=8$ ) ( $416 \pm 82$ ), while the TRX ( $n=7$ ) value was elevated ( $744 \pm 134$ ).

For epinephrine, PTU was again lower ( $287 \pm 68$ ) when compared with CON ( $484 \pm 129$ ), while TRX ( $774 \pm 189$ ) was elevated when compared with CON. Again,

circulating levels of EP were significantly increased in TRX when compared with CON and PTU levels.

When total catecholamine levels (NE + EP) were evaluated, TRX was again significantly increased ( $1817 \pm 437$ ) when compared to both CON ( $900 \pm 182$ ) and PTU ( $696 \pm 82$ ). Total catecholamine levels for the CON and PTU groups were not significantly different from each other.

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**Table 1:** BMR, systolic BP, and catecholamine analysis for each treatment group

Treatment Group	BMR cal/hr/m <sup>2</sup> ±SEM	Systolic BP mmHg±SEM	Norepinephrine pg/ml±SEM	Epinephrine pg/ml±SEM
CON (n=10)	◇# $58 \pm 3$	◇# $123.2 \pm 7.1$	◇ $416 \pm 82$	$484 \pm 129$
PTU (n=10)	*◇ $46 \pm 4$	*◇ $97.7 \pm 5.4$	◇ $394 \pm 71$	◇ $287 \pm 68$
TRX (n=10)	*# $76 \pm 4$	*# $163.2 \pm 8.5$	*# $744 \pm 134$	# $774 \pm 189$

\* denotes significance from CON at  $p < 0.05$

◇ denotes significance from TRX at  $p < 0.05$

# denotes significance from PTU at  $p < 0.05$

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### Phenylephrine-induced force in aortic ring segments

The force generated by the addition of PE and expressed in mg of force generated was plotted against the log molar PE for each sacrifice method. Figure 3 shows the results



when rats were sacrificed with pentobarbital anesthesia followed by thoracic opening. There is a general trend for force generated by PE in the order, TRX > CON > PTU. At the low PE dose, TRX ( $631.1 \pm 138.5$ ) was significantly greater when compared to CON ( $172.2 \pm 45.0$ ) and PTU ( $67.0 \pm 24.0$ ), however, the difference between CON and PTU was not statistically significant.

At the medium dose of PE, the trend in generated force persisted, with TRX ( $887.8 \pm 105.1$ ) significantly greater than PTU ( $193.0 \pm 57.5$ ). The force generated in CON ring segments at this dose was significantly greater than that produced by PTU rings. However, TRX was not significantly greater than CON ( $670.0 \pm 149.0$ ) at this level.

At high PE, the trend continued but the only significant difference in the force generated by PE was between TRX ( $1208.3 \pm 96.0$ ) and PTU ( $655.0 \pm 164.4$ ), with TRX being significantly greater. The high concentration of PE resulted in a generated force of  $970.0 \pm 100.0$  for CON.

When rats were sacrificed by cervical dislocation, responses to PE showed similar trends (Figure 4). The tendency again was for the general trend, TRX > CON > PTU, except at the lowest dose of PE. CON ( $401.3 \pm 151.3$ ) was significantly greater than PTU ( $33.8 \pm 7.3$ ) at this dose, while CON versus TRX ( $310.0 \pm 126.0$ ) was not significantly different.

At the medium PE dose, TRX ( $822.0 \pm 92.6$ ) was significantly greater than PTU ( $253.8 \pm 96.8$ ), but showed no significant difference from CON ( $848.8 \pm 159.7$ ). The PTU group was significantly lower than the CON mean at this dose.

At the high PE dose, there were no significant differences between the three treatment groups, however, the results continued to follow the general trend, TRX ( $927.1 \pm 76.7$ ) > CON ( $850.0 \pm 154.5$ ) > PTU ( $666.0 \pm 176.0$ ).

**Force unmasked by methylene blue addition to phenylephrine contracted aortic segments expressed in milligrams**

Log molar PE was plotted against the additional mg force generated upon exposure to MB in the presence of a PE-induced contraction for each sacrifice method (Figures 5 and 6). For pentobarbital anesthesia with thoracic opening (Figure 5) at the low PE dose, the increased tension in response to MB for CON ( $702.2 \pm 148.9$ ) was significantly greater than either TRX ( $270.0 \pm 62.0$ ) or PTU ( $266.0 \pm 84.3$ ). TRX and PTU were not significantly different from each other.

In rings contracted by the medium dose of PE, the resultant force increase with MB addition was  $635.0 \pm 130.2$  for CON, which was significantly greater than the increase seen for TRX ( $303.3 \pm 72.9$ ). CON and PTU ( $520.0 \pm 66.9$ ) were not significantly different from each other.

At the high PE concentration, MB addition produced additional force in PTU ( $598.8 \pm 127.3$ ) which was significantly greater than that seen in both the TRX ( $223.3 \pm 53.5$ ) and CON ( $333.9 \pm 64.3$ ) treatment groups. The CON and TRX groups were not significantly different from each other.

With cervical dislocation (Figure 6) at the low PE dose, none of the three groups was significantly different in their response to MB addition (CON,  $410.0 \pm 115.1$ ; TRX,  $463.0 \pm 124.2$ ; PTU,  $227.5 \pm 31.9$ ).

There were also no significant differences between the three treatment groups at the medium PE dose, (PTU,  $678.8 \pm 121.4$ ; TRX,  $405.0 \pm 59.9$ ; CON,  $657.5 \pm 148.7$ )

At the high concentration of PE, MB unmasked additional force in PTU ( $750.0 \pm 100.0$ ) to a significantly greater extent than in both CON ( $458.6 \pm 62.9$ ) and TRX ( $485.7 \pm 84.1$ ). The TRX group was not significantly different when compared with the CON group.

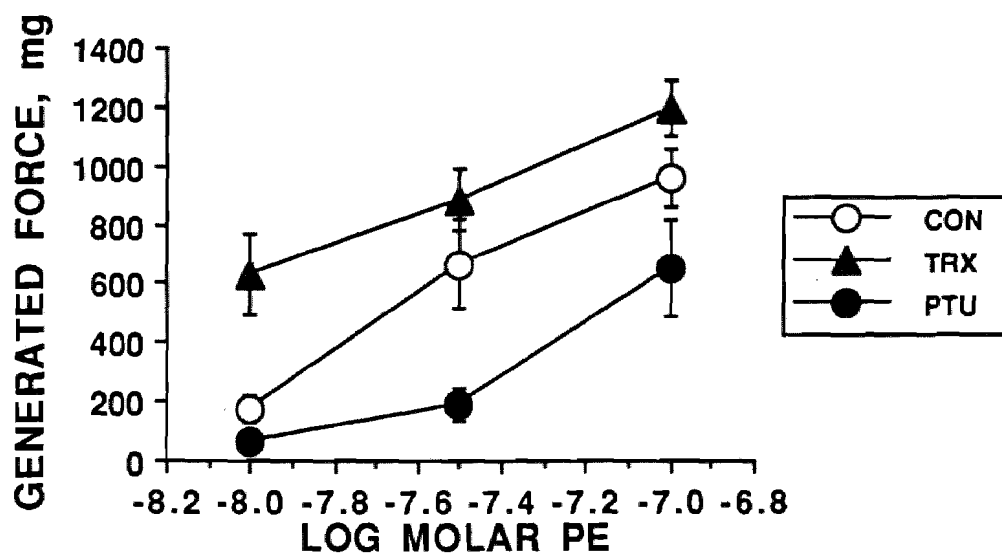


Figure 3. The force generated is plotted against the log molar PE concentration for rats sacrificed by pentobarbital anesthesia and thoracic opening.

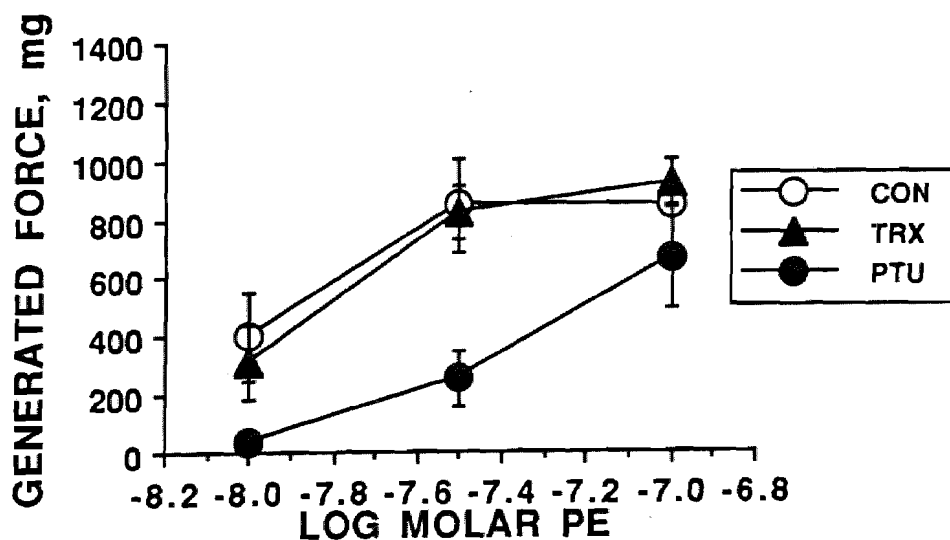


Figure 4. The force generated is plotted against the log molar PE concentration for rats sacrificed by cervical dislocation.

**Force unmasked by methylene blue addition to phenylephrine contracted aortic segments expressed as percent of total force generated by phenylephrine plus methylene blue**

The percent of maximum force generated by exposure to MB was plotted against the log molar PE for each method of sacrifice (Figures 7 and 8). Figure 7 shows the results when rats were sacrificed with pentobarbital anesthesia followed by thoracic opening. At the low PE dose, the trend showed increased force generation to MB in the order PTU > CON > TRX. CON ( $76.1 \pm 6.6$ ) and PTU ( $81.6 \pm 5.5$ ) were not significantly different, however, PTU showed a nonsignificant trend toward an increased force to MB. The force generated subsequent to MB addition for TRX ( $32.3 \pm 5.0$ ) was significantly decreased when compared to the responses to MB for both CON and PTU.

At the medium dose of PE, the trend was again PTU ( $75.4 \pm 4.1$ ) > CON ( $50.1 \pm 10.4$ ) > TRX ( $26.5 \pm 7.9$ ) for the additional force generated upon addition of MB. In this case, all three treatment groups were significantly different from each other.

At the high PE dose, the trend for MB addition expressed as a percent of the maximum force generated was again in the order PTU > CON > TRX. The PTU value ( $49.0 \pm 10.5$ ) was significantly greater than that for both TRX ( $15.6 \pm 3.5$ ) and CON ( $25.5 \pm 4.9$ ), while the TRX group was not significantly elevated when compared with CON.

Figure 8 shows the same measurements in response to MB when cervical dislocation was the method of sacrifice. While the same trend was seen in the order PTU > CON > TRX, there were fewer significant differences between the treatment groups. At the low PE dosage there were no significant differences (CON,  $58.7 \pm 12.6$ ; TRX,  $65.8 \pm 9.5$ ; PTU,  $86.6 \pm 3.5$ ).

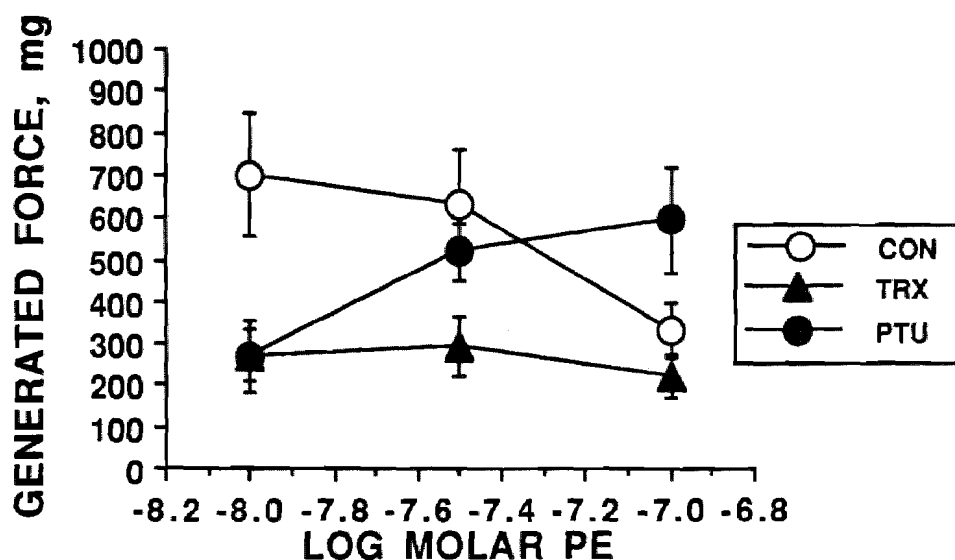


Figure 5. The contractile force prevented by EDNO release is plotted against the log molar PE concentration for rats sacrificed by anesthesia and thoracic opening (PE + MB-induced force minus PE-induced force).

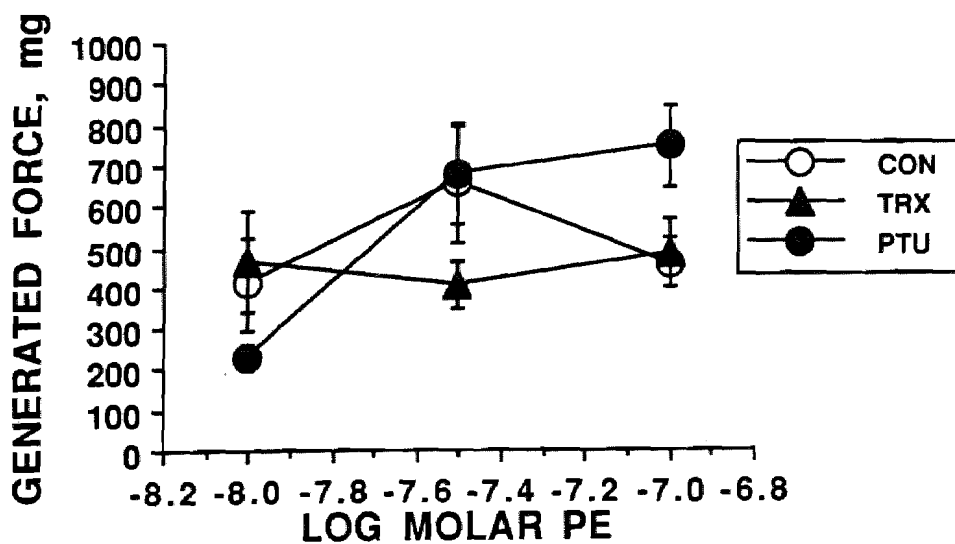


Figure 6. The contractile force prevented by EDNO release is plotted against the log molar PE concentration for rats sacrificed by cervical dislocation (PE + MB-induced force minus PE-induced force).

At the medium PE dose, CON ( $44.4 \pm 9.3$ ) versus PTU ( $74.3 \pm 8.1$ ) and TRX ( $34.6 \pm 7.1$ ) versus PTU showed significant differences while the difference between TRX and CON was not significant. However, the trend  $PTU > CON > TRX$  was again seen.

At the high PE dosage, the three treatment groups showed the same trend, PTU ( $55.1 \pm 9.8$ )  $>$  CON ( $38.8 \pm 8.3$ )  $>$  TRX ( $33.6 \pm 3.4$ ), however, there were no statistically significant differences.

#### **Effect of initial PE dose on force unmasked by methylene blue addition in euthyroid (CON) animals**

The percent of the force generated by PE plus MB addition that was due to the MB addition alone was plotted against the log molar PE concentration for CON rats sacrificed by the two different methods (Figures 7 and 8). Figure 7 shows the responses when rats were sacrificed by anesthesia and thoracic opening. The additional force generated by MB decreased as the initial PE dose increased. At the low dose of PE, the additional force generated by MB constituted  $76.1 \pm 6.6$  percent of the total PE plus MB contraction. The values at the medium and high PE doses were lower (medium dose,  $50.1 \pm 10.4$ ; high dose,  $25.5 \pm 4.9$ ). All three PE dose groups were significantly different from each other.

When rats were sacrificed by cervical dislocation, the results showed the same general trend, low dose ( $58.7 \pm 12.6$ )  $>$  medium dose ( $44.4 \pm 9.3$ )  $>$  high dose ( $38.8 \pm 8.3$ ), however, the differences were no longer significant (Figure 8).

#### **Pentobarbital anesthesia with thoracic opening versus cervical dislocation as method of sacrifice**

When results from the two methods of sacrifice were compared very few statistically significant differences were seen. CON and PTU rats of both sacrifice groups showed no significant differences in phenylephrine-induced force or in the additional MB-induced force, expressed in mg or as a percent of the maximum force generated (not

shown). The few differences found were between TRX animals sacrificed by the two different methods, however, there were no general trends or consistent differences.

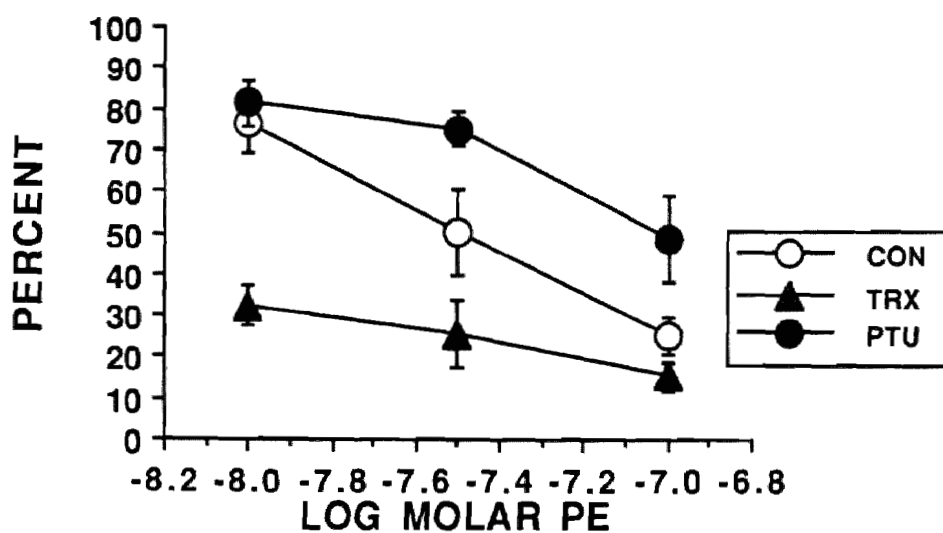


Figure 7. The percent of phenylephrine and methylene blue-induced force prevented by EDNO release is plotted against the log molar PE concentration for rats sacrificed by anesthesia and thoracic opening.

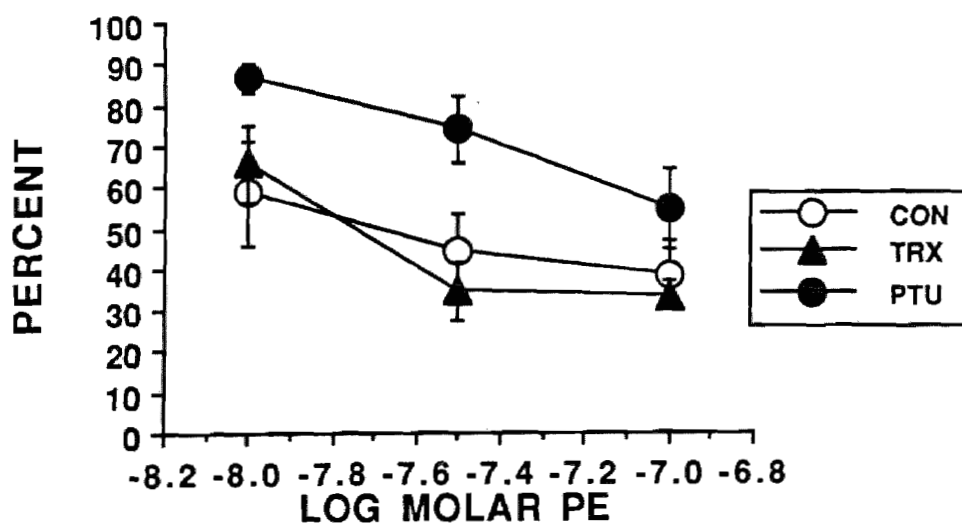


Figure 8. The percent of phenylephrine and methylene blue-induced force prevented by EDNO release is plotted against the log molar PE concentration for rats sacrificed by cervical dislocation



## DISCUSSION

Daily intraperitoneal injection of L-thyroxine was used to induce a hyperthyroid state in the TRX group. L-thyroxine elicits a hyperthyroid state by increasing the transcription of genes throughout the body, thereby increasing a number of structural proteins, enzymes, and transport proteins. The net effect is an increase in virtually all normal body activities and an increase in basal metabolic rate (BMR) (Guyton 1991; Klein 1990).

The hypothyroid state was induced in the PTU group by the addition of 2-thiouracil in the drinking water. 2-thiouracil acts within the thyroid cell to inhibit thyroperoxidase, the enzyme responsible for converting iodide to iodine and synthesizing thyroxine (T4) and triiodothyronine (T3) from diiodo- and moniodotyrosines (Guyton 1991). Therefore, the addition of 2-thiouracil prevents the synthesis of thyroid hormones and the animal is rendered hypothyroid with a decreased BMR. Previous studies in this laboratory have shown that when the thyroid gland is removed at time of sacrifice, there is decreased thyroid weight in hyperthyroid rats and increased thyroid weight in hypothyroid rats, a finding consistent with those thyropathologic states. Since BMR is increased in the hyperthyroid state and decreased in the hypothyroid state when compared to euthyroid rates, the appropriately altered BMRs in this study were taken as evidence of successful thyropathologic induction.

Systolic blood pressures were obtained using indirect tail cuff sphygmomanometry. This indirect method, when compared to direct measurements, has a correlation of almost one, making it a highly accurate, convenient, and noninvasive method for blood pressure determination (B.J. Sanders, personal communication). Previous studies have shown that systolic blood pressure is elevated in hyperthyroidism and lowered in hypothyroidism, when compared to euthyroid rats (Field et al. 1973; Rioux and Berkowitz 1977). Elevated

systolic pressures in hyperthyroidism may be due to an inability of the vascular network to accommodate the increase in cardiac output and stroke volume, or the effect of an increased blood volume (Klein 1990). In addition, increased norepinephrine acts on vessels to cause vasoconstriction and increased epinephrine acts on the heart to increase cardiac output (McDonough et al. 1987). Therefore, increased levels of these circulating catecholamines may contribute to the increased blood pressures observed in hyperthyroidism. In hypothyroid rats, there is a decreased heart rate and decreased cardiac output (McDonough et al. 1987). Accordingly, the elevated and depressed blood pressures measured in hyper- and hypothyroidism, respectively, are consistent with the systolic blood pressure changes known to accompany thyropathology and confirm indirectly the successful rendering of the thyropathologic states.

Previous studies in thyropathologic humans have found that circulating catecholamine levels were unchanged (Christensen 1973; Coulombe et al. 1976a, 1976b, 1977). The cardiovascular changes seen in those studies were therefore attributed to receptor density changes rather than to altered catecholamine levels. However, plasma catecholamine levels have not been reported for thyropathologic rats. The present study demonstrated increased circulating catecholamine levels in hyperthyroidism and decreased plasma levels in hypothyroidism. These changes in catecholamine levels presumably were due to increased sympathetic nervous system activity in hyperthyroidism and decreased sympathetic nervous system activity in hypothyroidism, though adrenoreceptor density may also be changed. The contributions of these changes in plasma catecholamine concentrations to changes in blood pressure, metabolic rate, and aortic reactivity were not further evaluated in this study.

Phenylephrine is a specific  $\alpha$ -1-adrenergic receptor agonist (Katzung, 1989). The force generated that was due to the initial application of PE showed a trend in the following order, TRX > CON > PTU, regardless of the initial PE dose. Previous studies

on the response to PE in thyropathology have been contradictory. No change, slight increases, or slight decreases in contractility have been reported in hypothyroidism (Coville and Telford 1970; Fox et al. 1985; Gunasekera and Kuriyama 1990; Hawthorn et al. 1988; Rahmani et al. 1987). In contrast to the present study, Coville and Telford (1970) and Fox et al. (1985) have reported inhibited vascular smooth muscle contractility to PE in the hyperthyroid state. However, Hawthorn et al. (1988) found that responses of isolated tail artery to PE were not significantly different in hyperthyroid rats. Differences between these studies could be due to different sacrifice methods, different treatment schedules, and different protocols. Further studies are needed to clarify the effect of thyropathology on rat aortic sensitivity to PE.

Receptor density changes in the thyropathologic states have also been reported recently. Gunasakera and Kuriyama (1990) reported a decrease in alpha-receptor density in hyperthyroidism and an increase in alpha-receptor density in hypothyroidism. Upon addition of PE, then, the hypothyroid state would be expected to show increased contractility while the hyperthyroid state would show a decreased response. However, Rahmani et al. (1987) found that in hypothyroid rat aortae, the alpha1-adrenoreceptor-mediated response was significantly decreased, and the maximum mechanical response was reduced. In contrast, hyperthyroid rat aortae showed no change in alpha-receptor-mediated responses. Gunasekera and Kuriyama (1990) found that the responses of alpha-adrenoreceptors to PE were markedly inhibited in the hypothyroid state, despite the increased number of alpha-adrenoreceptors. Gunasekera and Kuriyama (1990) also found that the hyperthyroid state resulted in decreased force development as seen in concentration-response curves when compared to the euthyroid rats. The present study also showed reduced contractility in the hypothyroid state in response to the alpha-adrenergic agonist PE, whereas, unlike the findings of Gunasekera and Kuriyama (1990), the hyperthyroid rat

aortae demonstrated increased contractility to a single PE dose. Again, further research is needed in this area.

The changes in response to PE may be related to altered blood pressure rather than to thyropathology *per se*. Increased contractility may be related to blood pressure increases which typically cause increased contractility and loss of endothelial function (Lockette et al. 1986; Lüscher 1990; Shimamura et al. 1991; Sunano et al. 1991). Since the hyperthyroid rats also developed a hypertensive condition, there may be an increased leakiness of stretch-activated calcium channels associated with hypertension (Rinaldi and Bohr 1989; Sunano et al. 1991) and a decreased synthesis and release of EDRFs in hypertension (Bohr et al. 1991; Lüscher et al. 1987; Lüscher 1990; Rees et al 1989; Shepherd and Katusic 1991; Sunano et al. 1991; Vanhoutte 1989; Wu et al. 1990). There may also be an increased release of contracting factors from the endothelium in hypertension (Bohr et al. 1991; Lüscher and Vanhoutte 1986; Lüscher 1990; Vanhoutte 1989) All of these factors would seem to cause an increased contractility in hypertension and therefore, possibly, in hyperthyroidism as well.

The release of EDRFs in response to acetylcholine was first discovered by Furchgott and Zawadzki (1980). Subsequently, a basal level of EDRF release was noted (Griffith et al. 1984; Lacolley et al. 1991; Long and Stone 1985; Rubanyi et al. 1985). More recently, VSM cells, themselves, have been shown to be another source of EDRFs (Wood et al. 1990).

Methylene blue, a soluble guanylate cyclase inhibitor, prevents the relaxing effect of NO on the VSM cell (Katsuki et al. 1977, Rappaport et al. 1985). Therefore, a ring precontracted with PE will develop additional force upon the addition of MB. This additional force represents the amount of force originally masked by basal NO release. It has been shown previously that contractile responses to PE are potentiated by MB (Carrier and White 1985; Ignarro et al. 1986). This study confirmed these findings in that MB

generated an additional force, even though the protocol was somewhat different. A similar protocol was used by Martin et al. (1986) except hemoglobin (Hb), another guanylate cyclase blocking agent, was added to rings already contracted by PE. They found that the addition of Hb consistently produced augmented force on rings already brought to maximal tone with PE. A subsequent study by Dainty et al. (1990), found that as the initial PE dose increased, the sensitivity to ACh decreased, indicating a reduced agonist-induced EDNO release. The results found in this study confirm, through a different protocol, the findings of these previous studies.

It was previously unknown what effect thyropathology would have on the MB contraction (basal NO release) or what effect the initial PE dose would have on the MB response. This study found that MB elicited an additional contraction in the order, PTU > CON > TRX, when values were expressed as a percent of the maximum force (PE + MB) generated. In other words, PTU rings had a greater total percentage of force uncovered by the MB while TRX rings generated less force upon MB addition when compared to CON. However, when the additional force generated by MB addition was expressed in mg, rather than as a percent of the total force, there were fewer significant differences between the thyropathologic states.

One explanation for these apparent differences in EDNO release could be the significantly different systolic blood pressures observed in the thyropathologic rats (hyperthyroid rats had significantly increased blood pressures while hypothyroid rats had significantly decreased systolic pressures). Many studies have found that the ability of vascular endothelial cells to synthesize or release EDRFs is attenuated in hypertension (Bohr 1991; Lüscher et al. 1987; Lüscher 1990; Rees et al. 1989; Shepherd and Katusic 1991; Sunano et al. 1991; Vanhoutte 1989; Wu et al. 1990). This study is consistent with the idea that hypertensive hyperthyroid rats may also demonstrate a reduced ability to synthesize or release EDNO. Although the effect of low blood pressure on EDNO

synthesis or release has not been studied, it could be hypothesized that hypotension might be accompanied by increased basal EDNO activity. Thus, the apparently increased release of EDNO in the PTU rats may be similarly explained, in part, by the decreased blood pressure that accompanies hypothyroidism.

The effect of the initial PE force on the additional force generated by MB was also previously unknown. This study found that as the initial PE-induced force increased, the force prevented by EDNO decreased in the euthyroid group, whether the force was expressed as a percent of maximum or in mg. Again, no previous studies were available for comparison, however, this finding seems appropriate. As the VSM cells are contracted to greater initial levels, there is no reason to assume that basal EDNO release would acutely change. Undoubtedly, there is a maximum level of force above which each ring cannot further contract. Therefore, as the PE-induced contraction begins to approach this maximal force, the MB becomes unable to increase the force further. This final possibility was disproved by the subsequent addition of a high PE dose which demonstrated that each ring had some residual ability to contract even after MB addition. Therefore, it seems likely that the decreased EDNO effect observed with increased initial force is a real phenomenon and not an artifact of the experimental design.

Vascular researchers evaluating thyropathology, alpha-adrenoreceptors, and endothelium-derived vasoactive factors routinely use different methods of rat sacrifice. It is important to ascertain whether different sacrifice methods might influence the results of a given study. Accordingly, another goal of this study was to compare vascular responses following the two most common but different methods of sacrifice: (1) anesthesia followed by thoracic opening, and (2) cervical dislocation with no anesthesia.

For the first sacrifice method, anesthesia followed by thoracic opening, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight) and then the thoracic cavity was opened and an incision made in the heart. Death

was due to either pneumothorax or exsanguination, but not due to anesthetic overdose. Rats have been routinely sacrificed by this and similar methods in previous studies (Lin and Nasjletti 1991; Lüscher and Vanhoutte 1986; Lüscher et al. 1987; Mortensen et al. 1990; Rinaldi and Bohr 1989; Wu and Bohr 1990). For the second method of sacrifice unanesthetized rats were killed by cervical dislocation to prevent any anesthesia effects. This method and similar methods have also been used in previous studies (Stratton and Morrow 1991; Dainty et al. 1990; Sufka et al. 1990).

When the two methods of sacrifice were compared, no differences in vascular response within the CON or PTU groups were observed. The TRX rats did demonstrate some significant differences depending on the method of sacrifice used. TRX rats killed by anesthesia and thoracic opening seemed to demonstrate augmented responses to PE as compared to TRX rats sacrificed by cervical dislocation at all three PE dose levels. However, the reason for this discrepancy is unclear. A potentially complicating factor is that sodium pentobarbital is a lipid soluble barbiturate (Katzung 1989) so the anesthetic may be retained by membranes within the aortic ring segments. In vivo, the tissue redistribution of a highly lipid soluble compound is facilitated by circulating lipoproteins and proteins, e.g. albumin, which have hydrophobic (lipophilic) cores. Since PSS is aqueous, two hours of equilibration may not be long enough to sufficiently remove the hydrophobic anesthetic from the ring preparations. Sodium pentobarbital is also known to reduce blood pressure and cerebral blood flow and to cause severe respiratory depression (Waynforth 1980). To reduce the effects of these complications, the rat aortae were removed as soon as full anesthesia was reached.

A complication of the second method of sacrifice (cervical dislocation with no anesthesia) is the stress which might be induced in the rat at the time of sacrifice. Cervical dislocation may be stressful to the rat because it is held by the tail while attempting to escape, and effective dislocation causes the expected muscle reflexes and spasms. This

process could result in increased catecholamine release whose effects may then be seen in the ring preparations and in the direct plasma catecholamine measurement. That is, massive sympathetic discharge at the time of sacrifice might down-regulate the number of alpha receptors in the vascular smooth muscle. Again, however, the time allowed for equilibration should have reduced or eliminated any such effects. Further studies are required to fully evaluate the effect of rat sacrifice method on vascular responses.



## CONCLUSIONS

From the data collected in the present study a number of conclusions can be reached. First of all, there seems to be altered circulating catecholamine levels depending on the thyropathologic state of the animal. Hyperthyroid animals demonstrated increased total plasma catecholamine levels while hypothyroid rats showed decreased levels when compared with euthyroid animals. Secondly, the basal release of EDNO was found to decrease with increasing initial PE concentrations in the CON animals sacrificed by anesthesia. Thirdly, the basal release of EDNO was found to be released differentially in thyropathology in the order, PTU > CON > TRX. Lastly, sacrifice method did not significantly alter results. Further studies evaluating the differences in protocol followed by different experimenters may clarify the contradictory findings of this study and those found within the literature.

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